ELSEVIER

Virus Research 89 (2002) 103–121

Virus Research

www.elsevier.com/locate/virusres

Functional conservation of HIV-1 Vpr and variability in a mother–child pair of long-term non-progressors

Yuqi Zhao [a,b,c,*], Mingzhong Chen [a], Bin Wang [d], June Yang [a], Robert T. Elder [a], Xiang-qian Song [a], Min Yu [a], Nitin K. Saksena [d]

[a] *Children's Memorial Institute for Education and Research, Northwestern University Feinberg School of Medicine, Chicago, IL, USA*
[b] *Department of Pediatrics, Northwestern University Feinberg School of Medicine, Chicago, IL, USA*
[c] *Departments of Microbiology-Immunology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA*
[d] *Retroviral Genetics Laboratory, Center for Virus Research, Westmead Millennium Institute, Westmead Hospital, University of Sydney, Westmead NSW 2145, Sydney, Australia*

Received 21 January 2002; received in revised form 12 June 2002; accepted 12 June 2002

Abstract

Increasing evidence suggests that HIV-1 Vpr is required in vivo for viral pathogenesis. Since Vpr displays multiple activities, little is known about which Vpr-specific activities are conserved in naturally occurring viruses or how natural mutations in Vpr might modulate viral pathogenesis in HIV-infected individuals. The goals of this study were to evaluate the functional variability of Vpr in naturally occurring viruses. The Vpr-specific activities of nuclear localization, induction of cell cycle G2 arrest and cell death were compared between viruses isolated from the fast progressing AIDS patients and a mother–child pair of long-term non-progressors (LTNPs). Wild-type Vpr activities were found in all of the viruses that were isolated from the fast progressing AIDS patients except for the truncated Vpr_{IIIB} which lacked these activities. In contrast, defective Vpr were readily detected in viral populations isolated, over an 11-year period, from the mother–child pair. Sequence analyses indicated that these Vpr carried unique amino acid substitutions that frequently interrupted a highly conserved domain containing an N-terminal α-helix-turn-α-helix. Thus, Vpr activities are generally conserved in naturally occurring viruses. The functionally defective Vpr identified in the mother–child pair of LTNPs are likely to be unique and may possibly contribute to the slow disease progression.

Keywords: HIV-1 Vpr; Nuclear translocation; Cell cycle G2 arrest; Cell death; Long-term non-progressors; AIDS; Fission yeast; *Schizosaccharomyces pombe*; HeLa

1. Introduction

Increasing evidence suggests that HIV-1 Vpr plays a pivotal role in viral pathogenesis, as its functions are being linked to viral activation (Levy et al., 1994), suppression of immune functions

* Corresponding author. Present address: 2430 N Halsted Street, MB218, Chicago, IL 60614, USA. Tel.: +1-773-880-6608; fax: +1-773-880-6609
E-mail address: yzhao@northwestern.edu (Y. Zhao).

0168-1702/02/$ - see front matter
PII: S0168-1702(02)00127-2

(Poon et al., 1998), and depletion of CD4 lymphocytes (Stewart et al., 1997). Vpr was also recently shown to be preferentially targeted by HIV-1-specific $CD8^+$ T-cells in comparison to other viral proteins during acute and latent infections, further indicating that Vpr is an important protein during HIV infection (Altfeld et al., 2001). Studies on chimpanzees and an accidentally infected laboratory worker demonstrated that Vpr is required in vivo for viral pathogenesis as the mutant *vpr* gene in the virus initiating the infection inevitably reverted back to the wild-type Vpr (Goh et al., 1998). Furthermore, when rhesus monkeys were infected with SIV defective in the *vpr*/*vpx* genes, the functional equivalent of the HIV-1 *vpr* gene, no disease progression was observed, unless the mutated Vpr reverted back to the wild type (Gibbs et al., 1995; Lang et al., 1993).

In vitro studies indicate that Vpr displays multiple activities, which include nuclear transport of the viral pre-integration complex (Heinzinger et al., 1994), cell cycle G2 arrest (Rogel et al., 1995), and induction of cell death (Stewart et al., 1997). These three effects of Vpr are independent activities of Vpr (Chen et al., 1999; Elder et al., 2000). The ability of Vpr to transport the viral pre-integration complex into the nucleus, which can be measured by the localization of Vpr to the nuclear envelope, is essential for viral entry to the nucleus and efficient infection of non-dividing macrophages (de Noronha et al., 2001; Heinzinger et al., 1994). The cell cycle G2 arrest induced by Vpr is thought to suppress human immune functions by preventing T-cell clonal expansion (Poon et al., 1998) and to provide an optimized cellular environment for maximal levels of viral replication (Goh et al., 1998). In addition, Vpr induces cell death, which may contribute to the depletion of CD4 T-cells in HIV-infected patients (Stewart et al., 1997).

Although Vpr has been recognized as one of the important viral factors in pathogenesis, the effect of naturally occurring *vpr* mutations on Vpr functions and their potential impact on disease progression of HIV-infected patients has not been reported. Earlier efforts to study the role of Vpr in viral pathogenesis and disease progression were mostly based on nucleotide sequencing analyses (Huang et al., 1998; Michael et al., 1995; Wang et al., 1996; Yedavalli and Ahmad, 2001; Zhang et al., 1997). Although DNA sequencing analysis is useful in detecting gene deletions or insertions, it does not determine whether amino acid substitutions cause functional changes. In fact, a number of mutagenesis studies have shown that a single amino acid substitution in Vpr can dramatically affect one or more activities of Vpr (Chen et al., 1999; Di Marzio et al., 1995; Yao et al., 1995; Zhou and Ratner, 2001). However, a potential problem in these mutagenesis studies is that the *vpr* mutants were artificially created and, therefore, may not represent the profile of naturally occurring mutations. Thus, the goal of this study was to examine the functional variability of Vpr activities in naturally occurring viruses and to assess the potential effects of this functional variation on disease progression in HIV-infected patients.

Three Vpr-specific activities, which include nuclear localization, cell cycle G2 arrest and cell killing, were examined in naturally occurring viruses and in commonly used HIV-1 viral strains that were originally isolated from the fast progressing AIDS patients. As a comparison, the same activities were also determined for Vpr isolated from a mother–child pair of long-term non-progressors (LTNPs) over a period of 11 years. This mother–child pair of LTNPs was chosen for this study because our sequence analyses showed that *vpr* genes isolated from these two patients were highly heterogeneous and had unique length polymorphisms, suggesting potential defects in Vpr (Wang et al., 1996). These Vpr-specific activities were examined in a fission yeast model system and were further validated in mammalian cells. These three Vpr activities observed in fission yeast are very similar to those observed in mammalian cells, and the use of fission yeast enables us to simultaneously examine multiple Vpr activities using an inducible expression system (Chen et al., 1999; Zhao et al., 1996, 1998a). This model system and its effective application to the study of Vpr-specific activities has been reviewed recently (Elder et al., 2002; Zhao and Elder, 2000).

2. Materials and methods

2.1. Patients and HIV-1 strains

Twelve HIV-1 viral strains (NL4-3, BAL, YU2, JRFL, JFCSF, SF162, 89.6, ELI, MAL, SF-2, LAI and IIIB) were obtained from the NIH AIDS Reagent Program. These well-characterized HIV-1 viral strains were all originally isolated from various tissues of AIDS patients including PBMC, cerebral spinal fluid (CSF), lung and brain. NL4-3 is a recombinant viral strain between NY5 and LAV, which were also isolated from AIDS patients (Adachi et al., 1986). All of these viral strains, except the IIIB strain, encode Vpr with a normal length of 96 amino acids (aa). The vpr_{IIIB} gene has a frame shift mutation at codon 73, which results in a truncated Vpr protein with only the first 72 aa (Popovic et al., 1984). For longitudinal studies, two fast progressing children, who were seen at the Chicago Children's Memorial Hospital, were chosen since they both consistently had high viral RNA load and low CD4 counts and met the clinical criteria for AIDS (C3) as defined by the CDC HIV classification system for children (1994). For comparison, a mother–child pair of LNTPs was chosen for this study because our previous sequence analyses showed that the *vpr* genes isolated from these two patients were highly heterogeneous and variable in length at the C-terminal end, suggesting potential functional defects (Wang et al., 1996). Both mother (LW) and child (JW), who are followed at the Westmead Hospital, Sydney, Australia, have survived more than 18 years with relatively stable CD4 T-cell counts and low viral RNA load as previously described (Wang, 2000; Wang et al., 1996). The mother was initially infected through a blood transfusion in 1983 and her child was subsequently infected via breast feeding at the end of 1983. No antiretroviral therapies were administered during the study period until 1997 when the mother started therapy and the child was treated briefly for 3 months. The HLA types of these two patients were determined by DNA sequencing done by the Children's Memorial Hospital Histocompatibility and Immunogenetics Laboratory.

2.2. Molecular cloning and characterization of the Vpr-specific activities

The *vpr* genes were initially isolated from uncultured PBMC of the infected patients and subsequently cloned by the standard limiting dilution method of PCR amplification into the cloning and expression vectors as previously described (Chen et al., 1999; Wang, 2000; Wang et al., 1996; Zhao et al., 1998a). Since a mixed *vpr* population was present in every clinical sample without a clearly predominant *vpr* species, 60 *vpr* clones were randomly isolated from the eight clinical samples collected from the mother and the nine samples collected from the child over the 11-year period from 1987 to 1998, and most of these sequences have been described previously (Wang, 2000; Wang et al., 1996). Twenty-two *vpr* clones of the mother–child pair, which represented unique amino acid substitutions of Vpr from various time points, were chosen for functional analysis in this study. For a complete description of the nucleotide sequences of *vpr* isolated from the mother–child pair, see Wang et al. (1996).

Four longitudinal samples of the uncultured PBMC, which were collected from each of the two fast progressing children over a period of 31 or 38 months, were subject to the Vpr functional characterization. To avoid the potential loss of minor *vpr* species, 18 random *vpr* clones from each patient were subject to the Vpr functional analysis prior to *vpr* gene sequencing analyses. To first determine whether these longitudinal samples carry a single predominant Vpr sequence overtime, a population sequencing approach was taken, i.e. the PCR product of the *vpr* gene was amplified directly from the PBMC and sequenced (Demeter et al., 1998; Yu et al., 2000). A single predominant Vpr sequence was observed in both patients over time. However, mixed *vpr* sequences were detected in three clinical samples. A shotgun cloning strategy was used to clone all the *vpr* genes detected by the population sequencing using standard limiting dilution method of PCR amplification. Ten random Vpr clones were subsequently sequenced for each sample to identify the unique Vpr sequences in the mixed *vpr* samples. This stepwise approach and subsequent sequen-

cing analyses led to the identification of two unique *vpr* sequences with one predominant and one minor sequence for the first patient (FP3254), and one predominant and two minor sequences for the second patient (FP4496).

The Vpr-specific activities were determined in fission yeast with an inducible gene expression system under the control of the *nmt1* (no message in thiamine) promoter (Maundrell, 1993; Zhao et al., 1996, 1998a). Under the control of this promoter, *vpr* expression can be either induced (*vpr*-on) or suppressed (*vpr*-off) simply by depleting or adding thiamine to the growth media (Zhao et al., 1996, 1998a). All of the functional assays used to measure Vpr-specific activities, i.e. cell cycle G2 arrest, nuclear localization and cell death induced by Vpr, have been described previously (Chen et al., 1999; Zhao et al., 1996, 1998b). Briefly, in order to quantify the extent of G2 arrest induced by Vpr, cells were taken from an active log phase thiamine-containing (*vpr*-off) culture (in the range of $1–5 \times 10^7$ cells per ml), washed three times with distilled water and used to start thiamine-minus (*vpr*-on) and thiamine-plus cultures at cell densities of 2×10^5 cell per ml at 30 °C with shaking (200 rpm). Cells were collected 40 h after the cultures were started, and the DNA content of the cells was determined by FACScan using Cell-fit software (Becton Dickinson). The extent of Vpr-induced G2 arrest was expressed as the percentage of G1 cells in the *vpr*-repressing cells that shifted to G2 phase of the cell cycle in *vpr*-expressing cells. The extent of cell cycle G2 arrest induced by *vpr* genes isolated from the patients was compared with the cell cycle profile of the wild-type Vpr_{NL4-3} reference strain (Fig. 1C, top left). A defect of a particular Vpr clone to block cell proliferation was further confirmed by analysis of growth kinetics (Fig. 1C, bottom; Zhao et al., 1996).

Colony forming ability was used to determine if a particular Vpr induces cell death as previously described (Zhao et al., 1998b). Briefly, *S. pombe* cells containing a pYZ1N::*vpr* construct were first grown on a selective leucine-free minimal EMM plate under *vpr*-repressing conditions. A loopful of viable cells was streaked onto *vpr*-inducing or *vpr*-repressing EMM plates and incubated at 30 °C for 3–4 days. Inability to form colonies on the *vpr*-inducing EMM plates but normal growth on the *vpr*-repressing EMM plates indicates Vpr-induced cell killing (Fig. 1D; Zhao et al., 1998b; Chen et al., 1999). Nuclear localization of Vpr was visualized with GFP-tagged Vpr (Fig. 1B). Cultures of cells with plasmids expressing GFP or the GFP–Vpr fusion proteins were prepared as described above for measurement of G2 arrest. Cellular localization was determined 18–24 h after *vpr* gene induction by fluorescence microscopy on a Leica microscope using the blue filter combination with the EY-455 supplemental exciter filter. Transfection of HeLa cells followed a procedure described previously (Stewart et al., 1997), and the cellular location of the GFP–Vpr fusion proteins was examined on a confocal microscope.

2.3. Amplification of full-length HIV-1 genome

PCR amplification of near full-length HIV-1 was done by Gene Amp XL PCR Kit (Perkin–Elmer) following the manufacture's protocol with the primer pair: UP (5′-TCTCTACG-CGTGGCGCCCGAA-3′) targeting the 5′-LTR and LOW (5′ ACCAGACGCGTACAACA-GACGGGCACACACTACTT-3′) targeting the 3′ LTR region. This primer pair produces a nearly full length HIV PCR product which includes part of the non-coding LTR and the coding regions of the *gag*, *pol*, *env gp160*, *nef*, *tat*, *rev*, *vpr*, *vpu* and *vif* genes. Since both the mother and child had very low copy numbers of proviral DNA, it was very difficult to amplify the full-length HIV genome directly from uncultured cells. Therefore, to minimize potential in vitro selection, instead of co-culturing with patient cells, we used PHA-stimulated cells and let them recover for 3 days in DMEM before PCR amplification. This full-length proviral DNA was further used as a template for genome walking and PCR amplification of individual viral genes. The PCR products were purified on the Millipore System and sequenced by di-deoxy chain termination method using gene-specific primer pairs. The intrinsic relationship between the two full-length viral genomes isolated from the mother and child was verified by phylogenetic analysis, and their unique

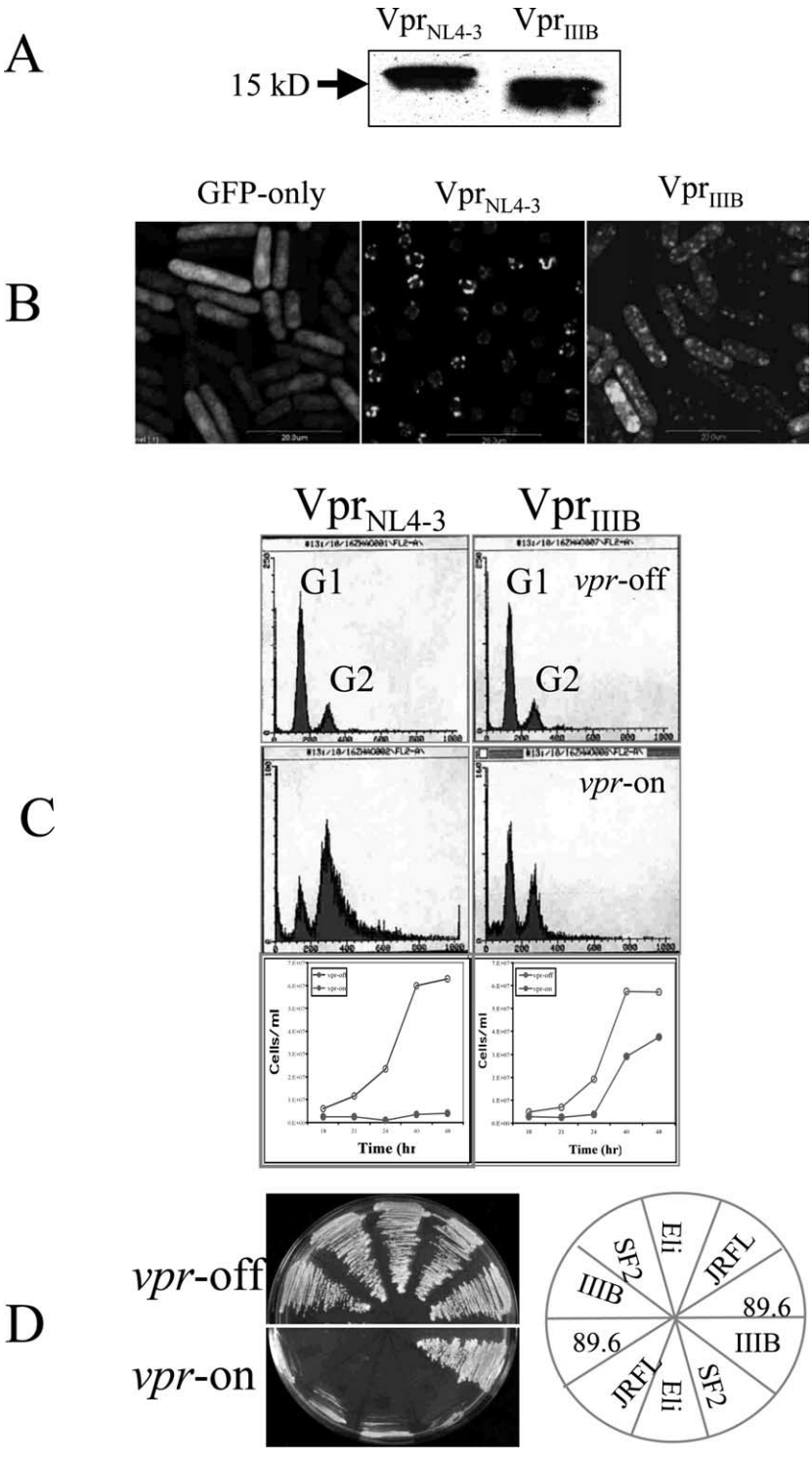
A
Vpr$_{NL4-3}$
Vpr$_{IIIB}$
15 kD
B
GFP-only
Vpr$_{NL4-3}$
Vpr$_{IIIB}$
C
Vpr$_{NL4-3}$
Vpr$_{IIIB}$
G1
G2
vpr-off
vpr-on
Cells/ml
Time (hr)
D
vpr-off
vpr-on
SF2
Eli
JRFL
89.6
IIIB

Fig. 1

Vpr sequences which were 98 amino acids in length (Fig. 3A) illustrates this relationship.

2.4. Nucleotide sequence alignment and phylogenetic analysis

Multiple HIV sequences from all patients were first manually screened and edited using Seqman II and Editseq. Multiple alignments of Vpr sequences were done using CLUSTAL V (Higgins et al., 1996) or JELLYFISH program (biowire.com). Phylogenetic analysis was performed with PHYLIP 3.5 (Felsenstein, 1988, 1992). The input sequences for isolates were randomized with the jumble option (where applicable). The phylogenetic tree to show intrinsic relationship between the mother and child of LTNPs was constructed with PRODIST (Dayhoff PAM matrix) and Neighbor. Reference sequences for phylogenetic analysis were obtained from the Los Alamos HIV Database (New Mexico, USA).

2.5. Viral cultures and measurement of viral replication in vitro

The preparation and infection of different cell types has been described previously (Kazazi et al., 1992; Wang, 2000). Briefly, for viral co-culture on PBMC, the patient's blood was collected and subjected to Ficoll–Hypaque gradient centrifugation at 1000 g for 10 min within 3 h of collection. PBMC were separated from plasma by centrifugation for 20 min. For coculture, 5×10^6 fresh PBMC were used with an equal number of uninfected PBMC which were stimulated with phytohemagglutinin (PHA-P; Sigma, St. Louis) and interleukin-2 (IL-2) for 48–72 h. The cultures were passaged twice each week with fresh PBMC.

In addition to passaging the virus into PBMCs, the virus from both mother and child was also passaged into monocytes and monocyte-derived macrophages (MDM) to measure viral replication kinetics. For viral culture on monocytes and MDM, the blood mononuclear cells from randomly chosen donors were first separated on Ficoll–Hypaque (Pharmacia, Sweden) and resuspended in 5 ml of elutriation medium (Hank's basal salt solution without Ca^{2+} or Mg^{2+}, 0.38% sodium citrate, and 0.4% bovine serum albumin). The cell suspension was separated into eight fractions by countercurrent elutriation in a Beckman J-6M/E centrifuge with a JE-5.0 elutriation rotor having 4.0 ml per chamber (Beckman instruments) as reported previously (Kazazi et al., 1992). Fractions 7 and 8 were harvested, and the purity of monocytes was checked by nonspecific esterase staining. The pooled fraction typically contained approximately 90% monocytes. Remaining T-lymphocytes were depleted by complement-dependent cytotoxicity, using a monoclonal anti-CD3 antibody (anti-OKT3; Ortho Diagnostics) and baby rabbit complement lysis. The resulting population was tested for residual T-cells by flow cytometry (Epics profile II; Coulter Electronics) and anti-CD3 monoclonal antibodies against a different epitope. Typically, 98–99% of purified cells were positive for nonspecific esterase, and $<1\%$ were positive for T-cells (Sigma kit no. 180). The infection of T-lymphocytes, monocytes and MDM was done using the PBMC expanded virus. A total of 2×10^6 adherent monocytes or MDMs were cultured in 24-well tissue cluster plates (Costar) in the Labtek dual-chamber slides in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and 10% heat-inactivated pooled human AB serum. Successful

Fig. 1. Conservation of Vpr activities in laboratory strains with the exception of Vpr_{IIIB}. (A) Vpr_{IIIB} is produced at the same level as other Vpr. Due to a frame shift at aa 72 introducing a termination codon after codon 78, expression of Vpr_{IIIB} produces a protein smaller than the normal size of 15 kD seen for Vpr from all other viral strains (only Vpr_{NL4-3} is shown). (B) Vpr_{IIIB} does not localize onto the nuclear envelope. Cellular localization of Vpr was assayed by expression of green fluorescent protein (GFP) in the left panel, of GFP fused to Vpr_{NL4-3} in the middle panel and GFP fused to Vpr_{IIIB} in the right panel. (C) Vpr_{IIIB} is defective in inducing cell cycle G2 arrest and in preventing cell proliferation. (D) Vpr_{IIIB} does not kill cells. The top-half of the picture is from a minimal EMM agar plate containing 20 μM thiamine to suppress *vpr* gene expression (*vpr*-off). The bottom half is from an EMM plate without thiamine to induce *vpr* expression (*vpr*-on). Expression of $Vpr_{89.6}$, Vpr_{JRFL}, Vpr_{Eli} and Vpr_{SF2} prevent colony formation, an indication of cell killing (Zhao et al., 1996) while expression of Vpr_{IIIB} does not prevent colony formation.

infection was confirmed by measuring extracellular HIV p24 antigen in culture supernatants and also by DNA PCR. During the experiments, special attention was taken to minimize technical factors as possible sources of variability. To achieve consistency, all cultures were treated identically, including aliquoting and storage of ultracentrifuged virus (to remove possible cytokine contamination) at −70 °C, infection at the same MOI [0.03 50% tissue culture infective dose (TCID50) per cell], aliquoting and storage of all culture supernatants at −70 °C for standardization, and carrying out the HIV antigen assays at the same time.

For kinetic studies of these infected cells, half of the cultured supernatant was collected and the culture was replenished with fresh medium every 3–4 days. Supernatant samples were stored at −70 °C for later assays. Viral replication in PBMC, T-lymphocytes, monocytes and MDM was assessed by measuring the p24 antigen levels in culture supernatants using an EIA kit (Coulter Immunology, Hialeeah, FL). The levels of HIV-1 p24 antigen in the culture supernatants were normalized for each viral strain and quantified by using the manufacturer's standards (HIV antigen assay; Abbott Laboratories, North Chicago, IL USA). The limit of detection of HIV p24 antigen was 9 pg/ml. When the upper limit of 300 pg/ml was exceeded, the samples were diluted with the kit diluent and re-assayed according to the manufacturer's recommendation.

3. Results

3.1. Conservation of HIV-1 Vpr activities in naturally occurring viruses and laboratory viral strains that were originally isolated from fast progressing AIDS patients

The protein sequence of Vpr appears to be the most conserved region of the HIV-1 viral genome with estimated similarities of 87% from all of the viruses isolated so far (Kuiken et al., 2000; Levy, 1998). It is still unclear, however, to what extent this 13% sequence variation affects the activities of Vpr. To examine this question, 12 well-characterized HIV-1 viral strains (NL4-3, BAL, YU2, JRFL, JFCSF, SF162, 89.6, ELI, MAL, SF-2, LAI and IIIB), which were originally isolated from various tissues (PBMC, CSF, lung and brain) of AIDS patients, were studied. The Vpr activities were measured in fission yeast cells as described previously (Chen et al., 1999; Zhao et al., 1996, 1998b). Nucleotide sequencing and immunoblot analyses confirmed that all these *vpr* genes were cloned and expressed properly in fission yeast cells (for an example with Vpr_{NL4-3} and Vpr_{IIIB}, see Fig. 1A).

These tests indicated that wild-type Vpr activities for nuclear localization, G2 arrest and cell death were observed in all of the viral strains except for the truncated Vpr_{IIIB} which lacked these activities. To ensure that the truncated Vpr_{IIIB} was produced at levels similar to other Vpr proteins, immunoblot analysis was performed with anti-Vpr serum which showed a strong protein band somewhat smaller than the expected full-length (15 kDa) Vpr proteins expressed from other *vpr* genes (Fig. 1A). Consistent with our previous observation that Vpr_{NL4-3} localizes onto the nuclear envelope (Chen et al., 1999), all GFP-Vpr fusion proteins except for Vpr_{IIIB} were restricted to the nuclear envelope (Fig. 1B). In contrast, Vpr_{IIIB} lost its ability to localize to the nuclear envelope with some of the protein aggregating in the cytoplasm. This pattern is different from the GFP protein by itself, which was dispersed evenly throughout the cells (Chen et al., 1999) (Fig. 1B). Equivalent levels of G2 arrest and cell killing were also observed for the Vpr from all of the viral strains with the exception of Vpr_{IIIB}. Vpr_{IIIB} failed to induce cell cycle G2 arrest and did not inhibit cell proliferation to the same extent as other Vpr proteins as indicated by cell cycle analysis and the measurement of growth kinetics (Fig. 1C). Furthermore, Vpr_{IIIB} did not induce cell death (Fig. 1D).

Measurement of Vpr activities for these HIV-1 viral strains suggested that Vpr activities are generally conserved. Since these viral strains have been adapted to the cell culture environment in the laboratory, however, the conservation of Vpr activities observed in these viral strains may not reflect what occurs during an HIV-1 infection. To

address this issue, a longitudinal study was carried out to examine the Vpr activities in naturally occurring HIV-1 viruses isolated from two fast progressing HIV-infected patients. These two fast progressors had consistently high viral load, low CD4 counts and a clinical status meeting the definition of AIDS over a period of 31 and 38 months (Table 1A). Nucleotide sequence analyses

Table 1

(A) Functional characterization of Vpr from a mother–child pair of LNTPs and two fast progressing children

Subject	Follow-up time (month)	Average viral RNA load±SD (log copies/ml)	Average CD4 Counts±SD (numbers/μl)	Average CD4/CD8 ratio	Vpr-specific functions (% of mutants)			Number of *vpr* clones examined (unique clones)	Total number of Vpr sequenced
					Nuclear transport	Cell cycle G2 arrest	Induction of cell death		
Long-term nonprogressors (LTNPs)									
LW (mother)	123	3.59±0.56	608±90	na	55%	27%	18%	11 (11)	30
JW (child)	132	3.58±0.41	674±102	na	45%	36%	27%	11 (11)	30
					50% **	**32% ****	**23% ***	22 (22)	60
Fast progressors (FPs)									
FP3254	38	5.65±0.41	176±133	0.17±0.10	0%	0%	0%	18 (2)	20
FP4496	31	5.57±0.54	148±94	0.12±0.05	0%	0%	0%	18 (3)	30
					0%	**0%**	**0%**	36 (5)	50

na, not available; Comparison between the long-term non-progressors and fast progressors with a p-value <0.001 (**) or <0.002 (*).

(B) Mutation profile of the functionally defective Vpr isolated from the mother–child pair of LTNPs

Amino Acid Sequence of HIV-1 Vpr

Amino acid 10 20 30 40 50 60 70 80 90	Length of Vpr (a.a.)	# a.a difference from consensus	Vpr Functions: Nuclear Localization	Vpr Functions: G2 Arrest	Vpr Functions: Cell Death
α - turn - α helix ; α helix					
Consensus MEQAPEDQG**P QREPYNEWAL ELLEELKNEA VRHFPRLWLH SLGQHIYETY** GDTWTGVEAI **IRILQQLLFI HFRIGC**QHSR IGITPQQRRG ARNGASRS	95-98		+	+	+
LW89-1 ---------- ---------- ---------- D--------- ---------- ---------- ---------- ---------- ---------_ --------	97	1	-	+	+
JW89-3 ---------- ---------- ---------- ---------- G--------- ---------- ---------- ---------- ------_--_ --------	96	1	-	+	+
LW94-2 ---------- ---------- ---------- ---------- ---------- -------GV- ---------- ---------- ------_--_ --------	96	2	-	+	+
JW97-3 ---------- ------S--- ---------- ---------- --R------- ---------- ---------- ---------- ------_--_ --------	96	2	-	+	+
LW97-2 ---------- ---------- K--------- ---------- ---------- ---------- ---------- ---------- T-----_--_ --------	96	2	-	+	+
JW94-1 ---------- ---------- ---------- ---------- ---R------ ---------- ---------- ---------- --V--_--_ --------	95	2	-	+	-
LW94-3 ---------- ---------- ---------- --R------- ---------- -------GV- ---------- ---------- --V---_--_ --------	96	3	-	-	+
LW95-1 ---------- ---------- --------G- ---------- ---------- ---------- ---------- ---------- ------_--_ --------	96	1	-	-	-
LW95-1182 ---------- ---------- ---------- ---------- --R----K-- -----R-K-- ---------- --K-----N- -----V---R --------	98	8	-	-	-
JW91-3 ---------- --xxxxxxxx	12		-	-	-
LW93-1 -------xxx xxxxxxxxxx	7		-	-	-

The X^2 test with binomial proportions showed statistically significant differences between the long-term non-progressors and fast progressors for all three V_{pr} activities with a $P < 0.001$ (**) for nuclear localization and G2 arrest and a $P < 0.002$ (*) for cell killing; na, not available.

The amino terminal α helix-turn-α-helix and the carboxyl terminal α helix are indicated (Wecker and Roques, 1999). Amino acids in bold represent the two conserved gap-less blocks (HIV sequence database, Los Alamos, NM). The underlined aa indicate where length polymorphism was found (Wang et al., 1996). –, identical aa; x, non-Vpr sequence due to frame shift; +, wild type phenotype; -, mutant phenotype.

of *vpr* clones from the PBMC of these two fast progressors showed that the length of Vpr was uniform (96 amino acids) with minimal sequence heterogeneity (Table 1A). Out of the 50 Vpr clones sequenced, only five unique Vpr variants were identified. Significantly, none of these five unique *vpr* variants identified in these two patients were defective for any of the three Vpr activities, suggesting that these natural sequence variations are wild-type for Vpr activities (Table 1A). Wild-type activities of Vpr isolated from these two fast progressing patients were further confirmed by measurements on a total of 36 random Vpr clones (Table 1A). Thus, no functionally defective Vpr could be detected in the total of eight samples taken from these two patients over a 31 or 38 month-period.

3.2. Functionally defective Vpr are frequently found in a mother–child pair of long-term non-progressors

In a parallel experiment, the same Vpr activities were also examined in the naturally occurring *vpr* genes isolated from the non-progressing mother–child pair described above (Table 1A). One unique feature of these Vpr populations was that the Vpr sequences were highly variable with no obvious predominant Vpr sequence in the viral populations over time (Wang et al., 1996). Moreover, unusually rare length polymorphisms of Vpr, which ranged from 95 to 98 amino acids in these samples, were also observed at the C-terminus of the protein, suggesting potential functional defects in Vpr (Wang et al., 1996). To examine systematically the functions of Vpr in the viruses infecting this long-term non-progressing mother and child pair, Vpr-specific activities were measured in 22 unique and longitudinally collected *vpr* clones. These unique *vpr* sequences represented a total of 60 random *vpr* gene clones derived from PBMC of the mother and child over a 123 and 132 month period, respectively, collected from 1987 to 1998 (Wang, 2000; Wang et al., 1996). Functional analyses of these naturally occurring *vpr* genes indicated that six out of 11 (55%) *vpr* clones examined from the mother (LW) and five out of 11 (45%) from the child (JW) were defective in their ability to localize onto the nuclear envelope (Table 1A). Approximately one third of the *vpr* clones from the mother and child had lost their ability to induce G2 arrest (32%, 7/22) and about one fourth (23%, 5/22) had lost the ability to induce cell death. Thus, variation of the *vpr* sequences in this pair of long term non-progressors does affect Vpr activities and there is a mixture of functionally active and defective Vpr in this pair of long term non-progressors.

3.3. Sequence analysis of Vpr variations and two full-length viral genomes isolated from the mother–child pair of LTNPs

To help distinguish sequence variation which does not affect Vpr activities from Vpr mutations which may affect Vpr activities, a consensus wild-type Vpr sequence was assembled by comparison of the 11 Vpr sequences with wild-type activity isolated from the child (JW87-1, JW91-4, JW91-5, JW93-1, JW95-1, JW97-1, and JW98-1) and the mother (LW87-2, LW87-5, LW89-3, and LW97-3) (Wang et al., 1996). Interestingly, Vpr could tolerate the length variation at the C-terminal end, as wild-type Vpr activities were observed in Vpr of all lengths ranging from 95 to 98 amino acids (Fig. 2A; Table 1B). In contrast, defects in Vpr functions were identified in Vpr clones that carry amino acid substitutions (Table 1B). For example, two *vpr* sequences isolated from the mother in 1994 and 1995 [JW94-1 (Table 1B) and JW95-1 (Fig. 2A)] differ by only two amino acids, Q44R and I83V, but distinctive Vpr activities were found for these two clones. While both clones induce G2 arrest at levels similar to those of the Vpr_{NL4-3} wild-type reference (Chen et al., 1999; Zhao et al., 1996), Vpr_{JW95-1} showed wild-type activities for nuclear localization and cell death, but Vpr_{JW94-1}, on the other hand, showed no cell killing and a significant reduction in its localization to the nuclear envelope (Fig. 2B and C).

To confirm that the Vpr activities observed in the fission yeast system accurately reflect the activities in mammalian cells, four *vpr* clones that showed either a wild-type or a mutant phenotype for nuclear localization from both the mother and child were tested in parallel in HeLa cells. Vpr_{NL4-3} was used as a positive control and

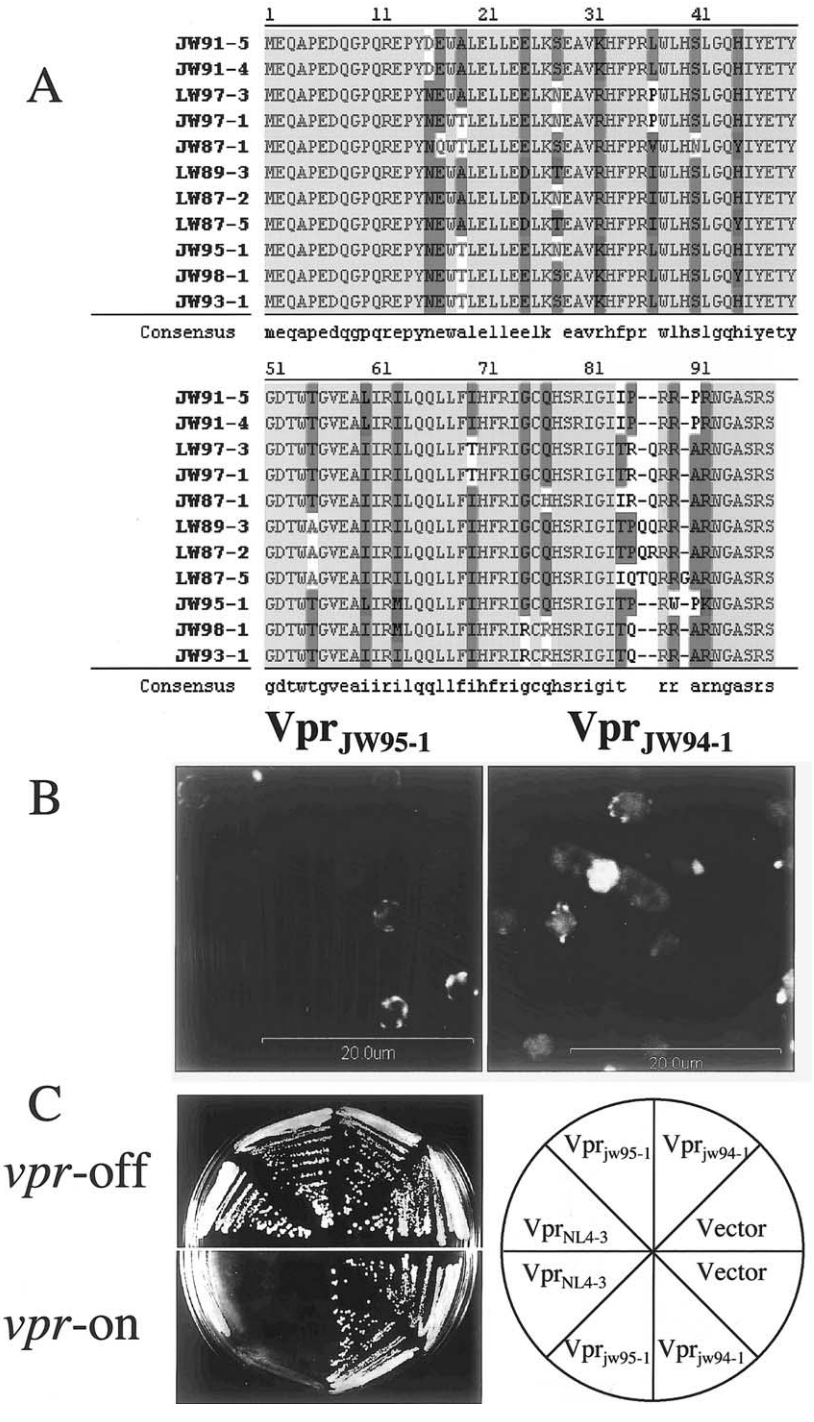
A
1 11 21 31 41
JW91-5 MEQAPEDQGPQREPYDEWALELLEELKSEAVKHFPRLWLHSLGQHIYETY
JW91-4 MEQAPEDQGPQREPYDEWALELLEELKSEAVKHFPRLWLHSLGQHIYETY
LW97-3 MEQAPEDQGPQREPYNEWALELLEELKNEAVRHFPRPWLHSLGQHIYETY
JW97-1 MEQAPEDQGPQREPYNEWTLELLEELKNEAVRHFPRPWLHSLGQHIYETY
JW87-1 MEQAPEDQGPQREPYNQWTLELLEELKSEAVRHFPRVWLHNLGQYIYETY
LW89-3 MEQAPEDQGPQREPYNEWALELLEDLKTEAVRHFPRIWLHSLGQHIYETY
LW87-2 MEQAPEDQGPQREPYNEWALELLEDLKNEAVRHFPRIWLHSLGQHIYETY
LW87-5 MEQAPEDQGPQREPYNEWALELLEDLKTEAVRHFPRIWLHSLGQYIYETY
JW95-1 MEQAPEDQGPQREPYNEWTLELLEELKNEAVKHFPRLWLHSLGQHIYETY
JW98-1 MEQAPEDQGPQREPYNEWTLELLEELKSEAVKHFPRLWLHSLGQYIYETY
JW93-1 MEQAPEDQGPQREPYNEWTLELLEELKSEAVKHFPRLWLHSLGQHIYETY
Consensus meqapedqgpqrepynewalelleelk eavrhfpr wlhslgqhiyety
51 61 71 81 91
JW91-5 GDTWTGVEALIRILQQLLFIHFRIGCQHSRIGIIP--RR-PRNGASRS
JW91-4 GDTWTGVEALIRILQQLLFIHFRIGCQHSRIGIIP--RR-PRNGASRS
LW97-3 GDTWTGVEAIIRILQQLLFTHFRIGCQHSRIGITR-QRR-ARNGASRS
JW97-1 GDTWTGVEAIIRILQQLLFTHFRIGCQHSRIGITR-QRR-ARNGASRS
JW87-1 GDTWTGVEAIIRILQQLLFIHFRIGCHHSRIGIIR-QRR-ARNGASRS
LW89-3 GDTWAGVEAIIRILQQLLFIHFRIGCQHSRIGITPQQRR-ARNGASRS
LW87-2 GDTWAGVEAIIRILQQLLFIHFRIGCQHSRIGITPQRRR-ARNGASRS
LW87-5 GDTWAGVEAIIRILQQLLFIHFRIGCQHSRIGIIQTQRRGARNGASRS
JW95-1 GDTWTGVEALIRMLQQLLFIHFRIGCQHSRIGITR--RW-PKNGASRS
JW98-1 GDTWTGVEAIIRMLQQLLFIHFRIRCRHSRIGITQ--RR-ARNGASRS
JW93-1 GDTWTGVEAIIRILQQLLFIHFRIRCRHSRIGITQ--RR-ARNGASRS
Consensus gdtwtgveaiirilqqllfihfrigcqhsrigit rr arngasrs
B
Vpr JW95-1
Vpr JW94-1
20.0um
20.0um
C
vpr-off
vpr-on
Vpr jw95-1
Vpr jw94-1
Vpr NL4-3
Vector
Vpr NL4-3
Vector
Vpr jw95-1
Vpr jw94-1

Fig. 2

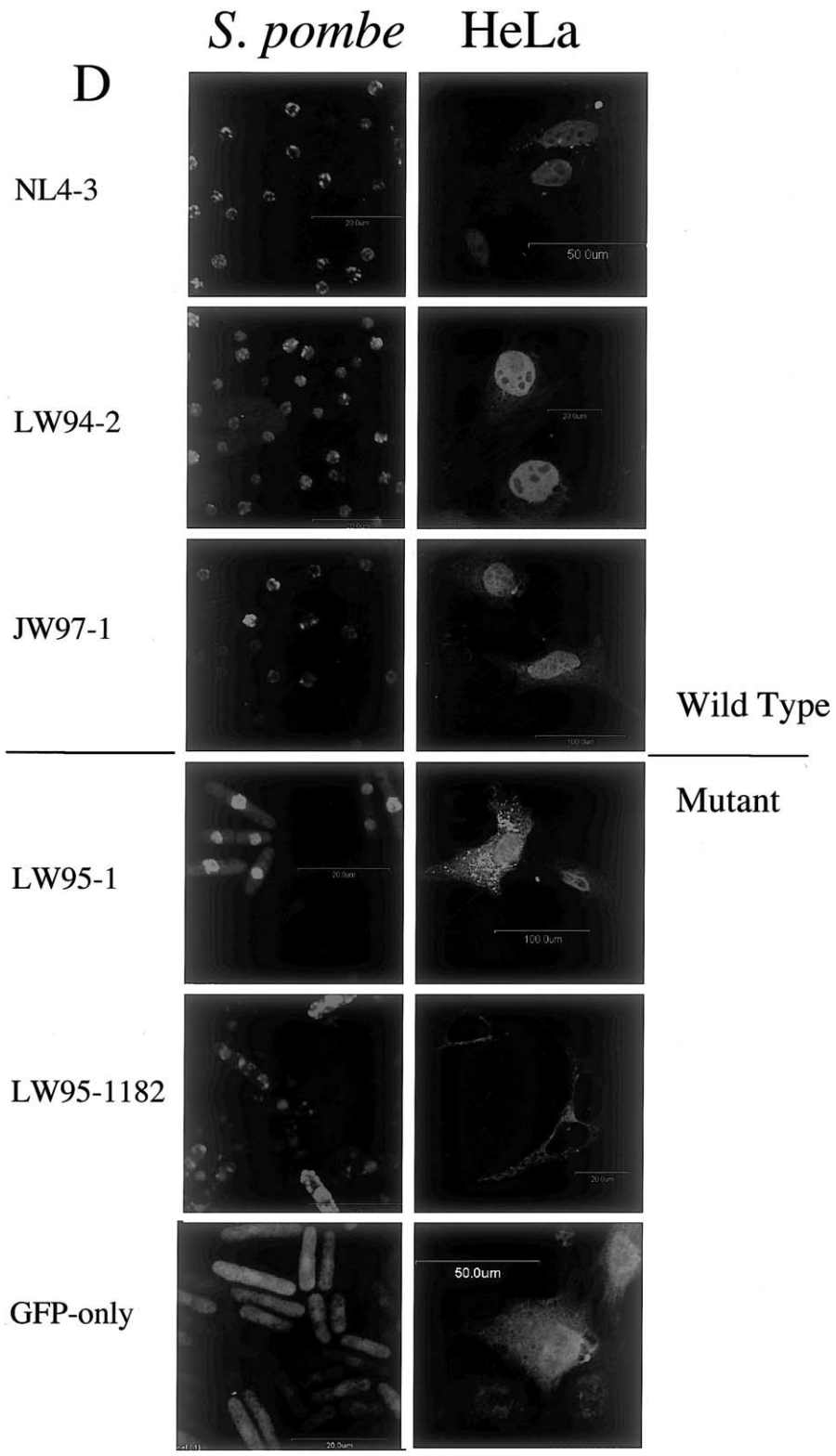

Fig. 2 (*Continued*)

GFP-alone as negative control. The patterns of nuclear localization of Vpr observed in fission yeast cells (Fig. 2D, left) were highly concordant with those observed in HeLa cells (Fig. 2D, right), even though GFP–Vpr does not exclusively localize onto the nuclear envelope in HeLa cells as it does in fission yeast. Thus, Vpr_{NL4-3}, Vpr_{LW94-2} and Vpr_{JW97-1} localized exclusively to the nuclear envelope in fission yeast, whereas they localized predominantly to the nucleus and nuclear periphery in HeLa cells. In contrast, the clone Vpr_{LW95-1} from the mother showed altered localization both in fission yeast and HeLa cells with localization in fission yeast throughout the nucleus and lesser amounts in the cytoplasm and the localization in HeLa cells throughout the nucleus and cytoplasm. Interestingly, clone $Vpr_{LW95-1182}$ showed no nuclear localization in fission yeast and was also completely excluded from the nucleus in HeLa cells.

Frequent detection of functionally defective Vpr in this mother–child pair of LTNPs suggests a possible association of these Vpr-defective viruses with the slow disease progression in this mother–child pair. However, other viral gene defects might also be present and contribute to the non-progression in these two patients. To address this issue, two complete viral genomes (9.8 kb) were isolated and sequenced from the PBMC taken in 1995 from the child (JW95-1181) and mother (LW95-1182), respectively (Wang, 2000). Overall, there was a good concordance in Vpr sequences between the mother and child for the nucleotide and amino acid sequences (Wang et al., 1996). When the full-length genomes (9.8 kb) were subjected to phylogenetic reconstruction using 100 bootstrap replications, the genomes from mother and child were closely related, thereby confirming the epidemiological-linkage and uniqueness of this mother–child pair (Fig. 3A). Nucleotide and amino acid sequence analyses of the full-length genomes showed no obvious gross gene defects, i.e. insertions/deletions or frame-shift mutations, in any of the other structural/regulatory/accessory genes with the exception of the *vpr* genes (Wang, 2000). The *vpr* genes isolated from the viruses infecting the mother and child encode a polypeptide of 98 aa in length, which is 2–3 aa longer than the average (Wang, 2000). Functional characterization of the $vpr_{LW95-1182}$ gene isolated from the mother indicated that it is defective in all three Vpr activities (Table 1B).

3.4. Two viral strains isolated from the mother–child pair do not efficiently replicate in different host cell types

Since Vpr activities have been linked to activation of viral replication and effective infection of monocyte and macrophages (Di Marzio et al., 1995; Levy et al., 1994), we were interested in finding out whether or not the Vpr-defective viruses isolated in the mother–child pair are also deficient in viral replication and infection of host cells. To test this hypothesis, the kinetics of viral replication were assessed by measuring the levels of extracellular viral p24 core antigen produced in culture by the viral strains isolated from the mother (LW95-1182) and child (JW95-1181) (Fig. 3B). Two commonly used laboratory HIV-1

Fig. 2. Molecular and functional analyses of Vpr isolated from the mother–child pair of LTNPs. (A) Alignment of amino acid sequences for Vpr that have wild-type activities. The aa sequences were aligned using a Multiple Sequence Alignment 'JELLYFISH' program (Biowire.com). The identical amino acids are in gray color. The evolutionary conserved residues are indicated by dark color. Consensus amino acids are listed under each residue. (B) Comparison of Vpr_{jw95-1} and Vpr_{jw94-1}. Both *vpr* genes were fused with GFP. GFP-Vpr_{jw95-1} localizes predominantly onto the nuclear rim (wild type). GFP-Vpr_{jw94-1} showed an abnormal localization pattern with reduced localization on the nuclear rim and a distribution to both the nucleus and cytoplasm. (C) Vpr_{jw94-1} does not kill cells (mutant) whereas Vpr_{jw95-1} does (wild type). The top-half of the picture is from a minimal EMM agar plate containing 20 μM thiamine to suppress *vpr* gene expression (*vpr*-off). The bottom half is from an EMM plate without thiamine to induce *vpr* expression (*vpr*-on). Vpr_{NL4-3} is used as a wild type control and a vector without *vpr* gene insert is used as a negative control. (D) The nuclear localization phenotype of Vpr is similar in fission yeast and HeLa cells. The GFP fusions to Vpr_{NL4-3}, Vpr_{LW94-2} from the mother or Vpr_{JW97-1} from the child show the wild-type nuclear localization phenotype with localization onto the nuclear envelope in fission yeast and throughout the nucleus in HeLa cells. Vpr_{LW95-1} and $Vpr_{LW95-1182}$, do not localize specifically to the nucleus in either fission yeast or HeLa cells. GFP by itself is distributed throughout the cell for both fission yeast and HeLa cells. Wt, wild type; Mt, mutant.

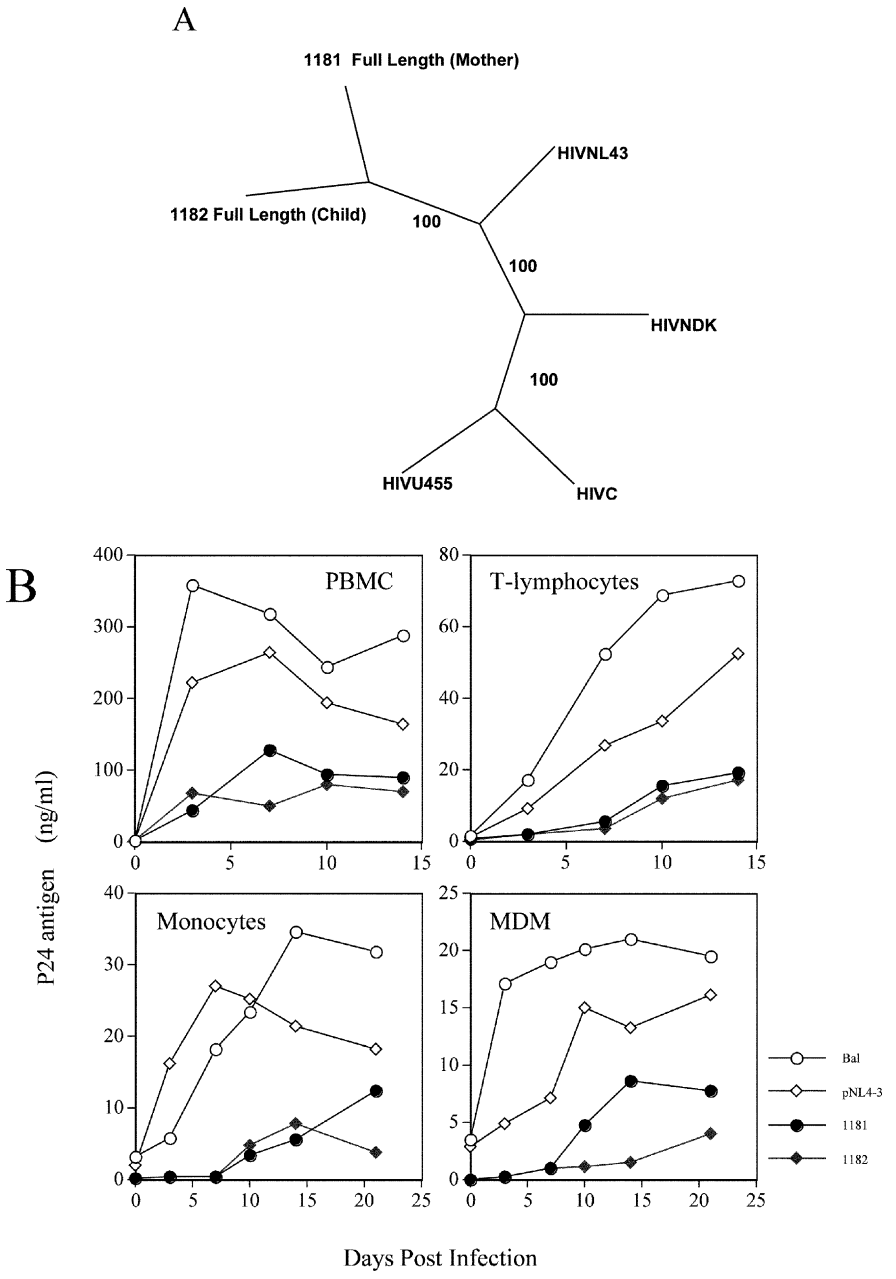

Fig. 3. Characterization of two HIV-1 viruses isolated from the mother–child pair of LTNPs. (A) HIV-1 viruses isolated from the mother–child pair are highly related but different from other reference strains. Based on the full-length viral genome, the neighbor-joining phylogenetic tree shows an absolute concordance between the HIV-1 genomes from the mother and child (LW-1181 and JW-1182). The other HIV-1 isolates which were used for phylogenetic comparisons are the representatives of subtype B (pNL43), subtype A/D recombinant (HIV NDK), HIV-1 subtype C (HIVC) and HIVU455. One hundred bootstrap replications were used for assessing the statistical concordance between HIV-1 from mother and child. (B) Replication kinetics of HIV-1 strains from mother and child on different cell types. HIV-1 viruses isolated from the mother–child pair replicate less efficiently than the wild-type Bal and pNL4-3 viral strains on PBMC, T-lymphocytes, monocytes, and MDM. Replication of these viral strains was normalized by p24 for comparison. –○–, Bal; –◇–, NL4-3; –●–, JW95-1181; –◆–, LW95-1182.

strains Bal and NL4-3, which have wild-type Vpr functions, were used as wild-type controls (Adachi et al., 1986; Gartner et al., 1986; Zhao et al., 1996). In comparison to the replication kinetics of the Bal and NL4-3 controls, both JW95-1181 and LW95-1182 showed lower levels of viral replication in PBMC (Fig. 3B, left top). Interestingly, these two viral strains did not cause any cytopathic effects, as determined by their non-syncytium inducing phenotype, even 14 days after infection of either PBMC or MT-2 cells. To test the ability of these two viral strains to replicate in different types of host cells, T-lymphocytes, monocytes and monocytes-derived macrophages (MDM) were infected with the same viral strains (Fig. 3B). Even though the viral strains from the mother and child were able to infect all three cell types, the levels of viral replication were consistently lower than the controls in all three cell types, suggesting that the viruses infecting the mother and child replicate less efficiently than wild-type viruses.

3.5. A highly conserved sequence block of Vpr was frequently interrupted by mutations in functionally defective Vpr from the mother–child pair of long-term non-progressors

The tertiary structure of Vpr based on NMR analyses (Wecker and Roques, 1999) contains an α-helix-turn-α-helix in the amino terminal half from aa 17–47 and an α-helix from aa 53–78 in the carboxy half of Vpr. Interestingly, early analyses of Vpr sequences ($N = 124$) deposited in the HIV database (Kuiken et al., 2000) revealed two highly conserved gap-less sequence blocks in Vpr. The first conserved sequence block (aa 9–48) overlaps the N-terminal α-helix-turn-α-helix, and the second block (aa 62–76) is within the C-terminal α-helix (boldfaced in Table 1B).

In order to understand the causal relationship between the naturally occurring *vpr* mutations and Vpr-specific activities, locations of the natural *vpr* mutations were correlated with the loss of a specific Vpr function. Since there is natural sequence variation in Vpr with wild-type activities, a consensus sequence for *vpr* with wild-type activities (Fig. 2A) was assembled from the eleven *vpr* sequences from the mother–child pair which have wild-type levels of all three activities, and this consensus sequence was compared with *vpr* clones deficient in one or more activities (Table 1B). We were interested in determining if the two highly conserved sequence blocks were frequently interrupted in the *vpr* clones that displayed mutant phenotypes. No mutations were found in the second conserved block with the exception of $Vpr_{LW95\text{-}1182}$, which has multiple amino acid substitutions (Table 1B). All of the Vpr mutants, except $Vpr_{LW94\text{-}2}$, carried mutation(s) within the first conserved block, and all had defective activities in nuclear localization (Table 1B). In addition, several interesting single amino acid substitutions were identified in the first conserved block that are likely to cause the specific mutant phenotypes of Vpr. For example, two single amino acid substitutions (V31D and S41G), identified in $Vpr_{LW89\text{-}1}$ and $Vpr_{JW89\text{-}3}$, are within the α-helix-turn-α-helix. Neither of these two mutant Vpr were able to localize onto the nuclear envelope but retained their ability to induce G2 arrest and cell killing, suggesting the change at aa 31 or 41 is solely responsible for the loss of nuclear localization (Table 1B). Interestingly, another single substitution at aa 29 (E29G) in $Vpr_{LW95\text{-}1}$ affected all three of the Vpr activities examined. Together, these data suggested that these particular *vpr* gene mutations isolated from the mother–child pair of LTNPs are unique in that they interrupt a normally highly conserved domain and are most likely responsible for the observed functional defects in Vpr.

4. Discussion

We show here that the Vpr activities of nuclear localization, cell cycle G2 arrest and induction of cell death are generally highly conserved in the naturally occurring viruses and the laboratory-adapted HIV-1 strains, which were originally isolated from rapidly progressing AIDS patients. Although functional conservation of Vpr in naturally occurring viruses has not been demonstrated previously, this finding is not surprising as the Vpr sequence is one of the most conserved regions of HIV-1 with an estimated similarity of 87% (Kui-

ken et al., 2000; Levy, 1998) and the *vpr* gene sequence is also conserved among HIV-1, HIV-2, SIV and other lentiviruses (Sharp et al., 1996; Tristem et al., 1992, 1998). Consistent with the conservation of Vpr, our data indicated that all of the amino acid variations of Vpr present in the twelve HIV-1 viral strains (except for Vpr_{IIIB}) and the two fast progressing AIDS patients do not change any of the three Vpr activities. These observations are consistent with the idea that Vpr is required in vivo for viral pathogenesis (Gibbs et al., 1995; Goh et al., 1998; Lang et al., 1993). However, the observation that some natural *vpr* mutations such as Vpr_{IIIB} are functionally defective for all three Vpr activities is very intriguing (Fig. 1A–D), as this is the same HIV-1 strain that was used to infect chimpanzees and was accidentally acquired by a laboratory worker (Goh et al., 1998). Since this frame-shift Vpr_{IIIB} mutation eventually reverted back to wild type in both the chimpanzees and the laboratory worker (Goh et al., 1998), we hypothesized that these three Vpr activities must be important for viral survival (Gibbs et al., 1995; Goh et al., 1998; Lang et al., 1993). Therefore, gene defects in *vpr* that diminish any of the Vpr-specific activities would have a negative impact on viral survival and thus could potentially slow down disease progression. To test this hypothesis, we carried out two longitudinal studies and compared the Vpr activities in naturally occurring viruses isolated from both fast and slow progressing HIV-infected patients. Consistent with our observations in the HIV-1 laboratory strains, Vpr activities were highly conserved in two fast progressing patients with no functional defects being detected in Vpr over a period of 31 or 38 months (Table 1A). In contrast, Vpr defective in nuclear localization, induction of G2 arrest and/or cell death were readily detected in a non-progressing mother–child pair over an 11-year period.

With regard to the mutant profile of Vpr, a highly conserved Vpr sequence block (aa 9–48) was revealed by analyses of all Vpr sequences deposited at the Los Alamos HIV-1 Database ($N = 124$). This conserved sequence block was frequently interrupted by gene mutations that are unique to this mother–child pair of LTNPs (Table 1B). The importance of this domain was further supported by the Vpr mutant profile in this region. For example, the substitution at position 31 (V31D) or 41 (S41G) within the α-helix-turn-α-helix was the only amino acid change between the wild-type Vpr consensus sequence and the mutant $Vpr_{LW89\text{-}1}$ or $Vpr_{JW89\text{-}3}$ (Table 1B). Neither of these two mutants were able to localize onto the nuclear envelope, suggesting the change at aa 31 or 41 is solely responsible for the loss of nuclear localization. These findings are consistent with previous observations that the N-terminal α-helix region is the primary structural domain for nuclear localization (Chen et al., 1999; Di Marzio et al., 1995). Significantly, the single substitution E29G in $Vpr_{LW95\text{-}1}$ affected all three of the Vpr activities examined. A similar finding was made previously for an artificially created single substitution H33R which also affected all three activities of Vpr (Chen et al., 1999). It is interesting that E29G and H33R occur in or immediately adjacent to the turn at aa 30–34 between the two amino terminal α helices suggesting that this region may be important in maintaining the overall structure of Vpr required for activity.

In vitro characterization of viral replication by the two viral isolates from the mother–child pair of LTNPs showed that replication in four different host cell types was significantly compromised (Fig. 3B). The presence of unique Vpr mutations and absence of other obvious gene defects in the nucleotide sequence of two viral genomes from the mother and child support a role for Vpr in the observed deficiency in viral replication (Di Marzio et al., 1995; Levy et al., 1994; Wang, 2000). However, these nucleotide sequence analyses do not rule out possible contributions from other viral gene defects to the observed replication deficiency. Another potential bias of this experiment is that the recovered viruses may have been adapted to the laboratory environment during the time required to culture the viral stocks required for these experiments. Nevertheless, the fact that both these viral isolates carried functionally defective Vpr and replicated much less efficiently than two commonly used laboratory-adapted viral strains indicate that they were either unable to replicate in vivo or replicated less efficiently even after adaptation in culture. Together, our data

suggest that the Vpr defects seen in the mother–child pair of LTNPs are unique in that they frequently disrupted a highly conserved and functionally important domain of Vpr and that these defects may contribute to the inefficient replication of these viruses.

In contrast to the gene reversion of mutated *vpr* in a HIV-infected laboratory worker and other primates (Gibbs et al., 1995; Goh et al., 1998; Lang et al., 1993), no obvious gene reversion of the mutated *vpr* were observed during the study period in the mother–child pair of LTNPs. In addition, a mixture of both functionally active and defective Vpr persisted in the viral populations without an obvious predominant Vpr sequence over the study period (Table 1B; Wang et al., 1996). Conversely, a single predominant Vpr sequence was evident in the two fast progressing patients even though minor Vpr sequences with wild-type activities were also detected in three samples. This finding is consistent with previous studies showing a positive correlation between viral homogeneity for either gp 120, Vpr or reverse transcriptase and disease progression (Wolinsky et al., 1996; Yedavalli and Ahmad, 2001). It was observed that the viral population is generally homogeneous in fast progressing patients whereas a more heterogeneous viral population often persists in patients with relatively slow disease progression. In many cases, it has been shown that this heterogeneity in a viral protein is related to CTL epitopes located in the protein with much of the variation representing escape mutations in the epitope (Allen et al., 2000; Kelleher et al., 2001; Moore et al., 2002). Thus, a diversified viral population may be associated with low viral load and reduced viral pathogenecity due to an effective CTL response by the host.

At least three possibilities could potentially explain how the wild type and mutant Vpr interact with each other and why heterogeneous Vpr populations persist in the mother–child pair of LTNPs over such a long time. The first possibility is that the mutant Vpr present in the mother–child pair of LTNPs is dominant over Vpr with wild-type activities. Even though this possibility is yet to be determined, this idea is, however, supported by a study showing that a mutant Vpr, which was defective in G2 arrest, was dominant over Vpr with the wild-type phenotype (Zhou and Ratner, 2001). Frequent switching of anti-retroviral therapies could also alter the composition of viral populations, but this possibility does not apply to these patients since neither the mother nor the child received any anti-retroviral treatment until the very end of the study period. Another possible explanation for the heterogeneity of Vpr could be that alteration of Vpr sequences in CTL epitope sequences may alleviate the host immune pressure on these Vpr-defective viruses, thus selecting for a diversified viral population containing escape mutations (Altfeld et al., 2001; Hughes et al., 2001) as has been demonstrated for other viral proteins (Wolinsky et al., 1996; Yedavalli and Ahmad, 2001). These Vpr-defective viruses which are under less immune pressure may then exist in a balance with viruses having functional Vpr but which are under stronger immune pressure. Consistently, Vpr is preferentially targeted for CTL attack (Altfeld et al., 2001). However, HLA typing of this mother–child pair of LNTP did not give a clear indication as to whether there are CTLs directed against Vpr in these patients. The HLA genotypes (LW A*2301, 11011 B*44031, 5501 and JW A*2301, 201 B*44031, 2705/2713) did not match any of the HLA types which are known to restrict Vpr epitopes (Altfeld et al., 2001). Interestingly, the B27 type has been associated with slow progression to AIDS (Hendel et al., 1999; Magierowska et al., 1999). Based on its binding properties (Rotzschke et al., 1994; Tanigaki et al., 1994), it has a number of predicted epitopes in Vpr, but this HLA type is present only in the child. For the shared haplotype of A*2301 B*44031, little information is available about the binding properties of A*2301, but the binding properties of B*4403 (Fleischhauer et al., 1994) suggest that there are potential epitopes in Vpr. Therefore, it will be necessary to examine the CTL responses in this mother–child pair of LTNP to determine if CTLs against Vpr play a role in the persistent functional and sequence heterogeneity of Vpr observed in these patients.

In summary, our data indicate that the Vpr-specific activities of nuclear localization, cell cycle G2 arrest and induction of cell death are highly

conserved in viruses derived from fast progressing AIDS patients. However, critical substitutions of a few, or even a single, amino acid of Vpr in naturally occurring viruses can have a significant impact on Vpr-specific activities. Since activities of Vpr are required in vivo during viral infection and are thought to have important roles in viral replication and pathogenesis (Connor et al., 1995; de Noronha et al., 2001; Di Marzio et al., 1995; Goh et al., 1998; Heinzinger et al., 1994), the functional defects of Vpr identified in this mother–child pair of LTNPs may be one of the viral factors contributing to their slow disease progression.

Acknowledgements

This study was supported in part by grants from Chicago Medical Research Institute Council (MRIC), MRIC Junior Board, Illinois Department of Public Aid, National Institute of Health AI40891 (YZ), and NHMRC grant 82030 (NKS). BW is a recipient of the IPRS Award and of a U2000 post-doctoral Fellowship Grant from the University of Sydney, and part of this study was from his dissertation in partial fulfillment of the requirements for the Doctor of Philosophy degree from the University of Sydney, Sydney, Australia. The authors wish to thank NIH AIDS Research and Reference Reagent Program for providing the HIV-1 laboratory strains, Susana G. Rodriguez-Marino of the Children's Memorial Hospital Histocompatibility and Immunogenetics Laboratory for the HLA typing, Tina Chen for assistance on statistical analyses and Kuntal Shah for initial Vpr sequence analyses.

References

Adachi, A., Gendelman, H.E., Koenig, S., Folks, T., Willey, R., Rabson, A., Martin, M.A., 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. 59, 284–291.

Allen, T.M., O'Connor, D.H., Jing, P., Dzuris, J.L., Mothe, B.R., Vogel, T.U., Dunphy, E., Liebl, M.E., Emerson, C., Wilson, N., Kunstman, K.J., Wang, X., Allison, D.B., Hughes, A.L., Desrosiers, R.C., Altman, J.D., Wolinsky, S.M., Sette, A., Watkins, D.I., 2000. Tat-specific cytotoxic T-lymphocytes select for SIV escape variants during resolution of primary viraemia. Nature 407, 386–390.

Altfeld, M., Addo, M.M., Eldridge, R.L., Yu, X.G., Thomas, S., Khatri, A., Strick, D., Phillips, M.N., Cohen, G.B., Islam, S.A., Kalams, S.A., Brander, C., Goulder, P.J., Rosenberg, E.S., Walker, B.D., 2001. Vpr Is Preferentially Targeted by CTL During HIV-1 Infection. J. Immunol. 167, 2743–2752.

Chen, M., Elder, R.T., Yu, M., O'Gorman, M., Selig, L., Benarous, R., Yamamoto, A., Zhao, Y., 1999. Mutational analysis of Vpr-induced G2 arrest, nuclear localization and cell death in fission yeast. J. Virol. 73, 3236–3245.

Connor, R.I., Chen, B.K., Choe, S., Landau, N.R., 1995. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology 206, 935–944.

de Noronha, C.M., Sherman, M.P., Lin, H.W., Cavrois, M.V., Moir, R.D., Goldman, R.D., Greene, W.C., 2001. Dynamic disruptions in nuclear envelope architecture and integrity induced by HIV-1 Vpr. Science 294, 1105–1108.

ACTG Sequencing Working Group. AIDS Clinical Trials Group, Demeter, L.M., D'Aquila, R., Weislow, O., Lorenzo, E., Erice, A., Fitzgibbon, J., Shafer, R., Richman, D., Howard, T.M., Zhao, Y., Fisher, E., Huang, D., Mayers, D., Sylvester, S., Arens, M., Sannerud, K., Rasheed, S., Johnson, V., Kuritzkes, D., Reichelderfer, P., Japour, A., 1998. Interlaboratory concordance of DNA sequence analysis to detect reverse transcriptase mutations in HIV-1 proviral DNA. J. Virol. Methods 75, 93–104.

Di Marzio, P., Choe, S., Ebright, M., Knoblauch, R., Landau, N.R., 1995. Mutational analysis of cell cycle arrest, nuclear localization and virion packaging of human immunodeficiency virus type 1 Vpr. J. Virol. 69, 7909–7916.

Elder, R.T., Yu, M., Chen, M., Edelson, S., Zhao, Y., 2000. Cell cycle G2 arrest induced by HIV-1 Vpr in fission yeast (*Schizosaccharomyces pombe*) is independent of cell death and early genes in the DNA damage checkpoint. Virus Res. 68, 161–173.

Elder, R.T., Benko, Z., Zhao, Y., 2002. HIV-1 Vpr modulates cell cycle G2/M transition through an alternative cellular mechanism other than the classic mitotic checkpoints. Front Biosci. 7, 349–357.

Felsenstein, J., 1988. Phylogenies from molecular sequences: inference and reliability. Annu. Rev. Genet. 22, 521–565.

Felsenstein, J., 1992. Estimating effective population size from samples of sequences: inefficiency of pairwise and segregating sites as compared with phylogenetic estimates. Genet. Res. 59, 139–147.

Fleischhauer, K., Avila, D., Vilbois, F., Traversari, C., Bordignon, C., Wallny, H.J., 1994. Characterization of natural peptide ligands for HLA-B*4402 and -B*4403: implications for peptide involvement in allorecognition of a single amino acid change in the HLA-B44 heavy chain. Tissue Antigens 44, 311–317.

Gartner, S., Markovits, P., Markovitz, D.M., Kaplan, M.H., Gallo, R.C., Popovic, M., 1986. The role of mononuclear phagocytes in HTLV-III/LAV infection. Science 233, 215–219.

Gibbs, J.S., Lackner, A.A., Lang, S.M., Simon, M.A., Sehgal, P.K., Daniel, M.D., Desrosiers, R.C., 1995. Progression to AIDS in the absence of a gene for *vpr* or *vpx*. J. Virol. 69, 2378–2383.

Goh, W.C., Rogel, M.E., Kinsey, C.M., Michael, S.F., Fultz, P.N., Nowak, M.A., Hahn, B.H., Emerman, M., 1998. HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo. Nature Med. 4, 65–71.

Heinzinger, N.K., Bukinsky, M.I., Haggerty, S.A., Ragland, A.M., Kewalramani, V., Lee, M.A., Gendelman, H.E., Ratner, L., Stevenson, M., Emerman, M., 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc. Natl. Acad. Sci. USA 91, 7311–7315.

Hendel, H., Caillat-Zucman, S., Lebuanec, H., Carrington, M., O'Brien, S., Andrieu, J.M., Schachter, F., Zagury, D., Rappaport, J., Winkler, C., Nelson, G.W., Zagury, J.F., 1999. New class I and II HLA alleles strongly associated with opposite patterns of progression to AIDS. J. Immunol. 162, 6942–6946.

Higgins, D.G., Thompson, J.D., Gibson, T.J., 1996. Using CLUSTAL for multiple sequence alignments. Methods Enzymol. 266, 383–402.

Huang, Y., Zhang, L., Ho, D.D., 1998. Characterization of gag and pol sequences from long-term survivors of human immunodeficiency virus type 1 infection. Virology 240, 36–49.

Hughes, A.L., Westover, K., da Silva, J., O'Connor, D.H., Watkins, D.I., 2001. Simultaneous positive and purifying selection on overlapping reading frames of the *tat* and *vpr* genes of simian immunodeficiency virus. J. Virol. 75, 7966–7972.

Kazazi, F., Mathijs, J.M., Chang, J., Malafiej, P., Lopez, A., Dowton, D., Sorrell, T.C., Vadas, M.A., Cunningham, A.L., 1992. Recombinant interleukin 4 stimulates human immunodeficiency virus production by infected monocytes and macrophages. J. Gen. Virol. 73, 941–949.

Kelleher, A.D., Long, C., Holmes, E.C., Allen, R.L., Wilson, J., Conlon, C., Workman, C., Shaunak, S., Olson, K., Goulder, P., Brander, C., Ogg, G., Sullivan, J.S., Dyer, W., Jones, I., McMichael, A.J., Rowland-Jones, S., Phillips, R.E., 2001. Clustered mutations in HIV-1 gag are consistently required for escape from HLA-B27-restricted cytotoxic T-lymphocyte responses. J. Exp. Med. 193, 375–386.

Kuiken, C., Foley, B., Hahn, B.H., Marx, P., McCutchan, F., Mellors, J., Mullins, J., Sodroski, J., Wolinsky, S.M., Korber, B., 2000. HIV Sequence Compendium, second ed.. Los Alamos National Laboratory, p. 592.

Lang, S.M., Weeger, M., Stahl-Hennig, C., Coulibaly, C., Hunsmann, G., Muller, J., Muller-Hermelink, H., Fuchs, D., Wachter, H., Daniel, M.M., Desrosiers, R.C., Fleckenstein, B., 1993. Importance of *vpr* for infection of rhesus monkeys with simian immunodeficiency virus. J. Virol. 67, 902–912.

Levy, J.A., 1998. HIV and the Pathogenesis of AIDS, second ed.. ASM Press, p. 588.

Levy, D.N., Refaeli, Y., MacGregor, R.R., Weiner, D.B., 1994. Serum Vpr regulates productive infection and latency of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA 91, 10873–10877.

Magierowska, M., Theodorou, I., Debre, P., Sanson, F., Autran, B., Riviere, Y., Charron, D., Costagliola, D., 1999. Combined genotypes of CCR5, CCR2, SDF1, and HLA genes can predict the long-term nonprogressor status in human immunodeficiency virus-1-infected individuals. Blood 93, 936–941.

Maundrell, K., 1993. Thiamine-repressible expression vectors pREP and pRIP for fission yeast. Gene 123, 127–130.

Michael, N.L., Chang, G., d'Arcy, L.A., Ehrenberg, P.K., Mariani, R., Busch, M.P., Birx, D.L., Schwartz, D.H., 1995. Defective accessory genes in a human immunodeficiency virus type 1-infected long-term survivor lacking recoverable virus. J. Virol. 69, 4228–4236.

Moore, C.B., John, M., James, I.R., Christiansen, F.T., Witt, C.S., Mallal, S.A., 2002. Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. Science 296, 1439–1443.

Poon, B., Grovit-Ferbas, K., Stewart, S.A., Chen, I.S.Y., 1998. Cell cycle arrest by Vpr in HIV-1 virions and insensitivity to antiretroviral agents. Science 281, 266–269.

Rogel, M.E., Wu, L.I., Emerman, M., 1995. The human immunodeficiency virus type 1 *vpr* gene prevents cell proliferation during chronic infection. J Virol. 69, 882–888.

Rotzschke, O., Falk, K., Stevanovic, S., Gnau, V., Jung, G., Rammensee, H.G., 1994. Dominant aromatic/aliphatic C-terminal anchor in HLA-B*2702 and B*2705 peptide motifs. Immunogenetics 39, 74–77.

Sharp, P.M., Bailes, E., Stevenson, M., Emerman, M., Hahn, B.H., 1996. Gene acquisition in HIV and SIV. Nature 383, 586–587.

Stewart, S.A., Poon, B., Jowett, J.B., Chen, I.S., 1997. Human immunodeficiency virus type 1 Vpr induces apoptosis following cell cycle arrest. J. Virol. 71, 5579–5592.

Tanigaki, N., Fruci, D., Vigneti, E., Starace, G., Rovero, P., Londei, M., Butler, R.H., Tosi, R., 1994. The peptide binding specificity of HLA-B27 subtypes. Immunogenetics 40, 192–198.

Tristem, M., Purvis, A., Quicke, D.L., 1998. Complex evolutionary history of primate lentiviral *vpr* genes. Virology 240, 232–237.

Tristem, M., Marshall, C., Karpas, A., Hill, F., 1992. Evolution of the primate lentiviruses: evidence from *vpx* and *vpr*. EMBO J. 11, 3405–3412.

Wang, B., 2000. Biological and Molecular Mechanisms in HIV Disease Progression and Co-infection. Ph.D. dissertation. University of Sydney, Australia pp. 85–112 (Chapter 3).

Wang, B., Ge, Y., Palasanthiran, P., Xiang, S., Ziegler, J., Dwyer, D., Randle, C., Dowton, D., Cunningham, A., Saksena, N., 1996. Gene defects clustered at the C-terminus

of the *vpr* gene of HIV-1 in long-term nonprogressing mother and child pair: in vivo evolution of *vpr* quasispecies in blood and plasma. Virology 223, 224–232.

Wecker, K., Roques, B.P., 1999. NMR structure of the (1-51) N-terminal domain of the HIV-1 regulatory protein Vpr. Eur. J. Biochem. 266, 359–369.

Wolinsky, S.M., Korber, B.T., Neumann, A.U., Daniels, M., Kunstman, K.J., Whetsell, A.J., Furtado, M.R., Cao, Y., Ho, D.D., Safrit, J.T., 1996. Adaptive evolution of human immunodeficiency virus-type 1 during the natural course of infection. Science 272, 537–542.

Yao, X.J., Subbramanian, R.A., Rougeau, N., Boisvert, F., Bergeron, D., Cohen, E.A., 1995. Mutagenic analysis of human immunodeficiency virus type 1 Vpr: role of a predicted N-terminal α-helical structure in Vpr nuclear localization and virion incorporation. J. Virol. 69, 7032–7044.

Yedavalli, V.R., Ahmad, N., 2001. Low conservation of functional domains of HIV Type 1 *vif* and *vpr* genes in infected mothers correlates with lack of vertical transmission. AIDS Res. Hum. Retroviruses 17, 911–923.

Yu, M., Kabat, W., Wang, J.W., Hung, T., Yogev, R., Zhao, Y., 2000. Rapid detection of drug resistant HIV -1 to reverse transcriptase and protease inhibitors in HIV-infected patients receiving highly active antiretroviral therapies. Chin. J. Exp. Clin. Virol. (Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi) 14, 325–329.

Zhang, L., Huang, Y., Yuan, H., Tuttleton, S., Ho, D.D., 1997. Genetic characterization of *vif*, *vpr*, and *vpu* sequences from long-term survivors of human immunodeficiency virus type 1 infection. Virology 228, 340–349.

Zhao, Y., Elder, R.T., 2000. Yeast perspectives on HIV-1 Vpr. Front Biosci. 5, 905–916.

Zhou, Y., Ratner, L., 2001. A novel inducible expression system to study transdominant mutants of HIV-1 vpr. Virology 287, 133–142.

Zhao, Y., Cao, J., O'Gorman, M.R.G., Yu, M., Yogev, R., 1996. Effect of human immunodeficiency virus Type 1 protein R (*vpr*) gene expression on basic cellular functions of fission yeast *Schizosaccharomyces pombe*. J. Virol. 70, 5821–5826.

Zhao, Y., Elder, R.T., Chen, M., Cao, J., 1998a. Fission yeast expression vectors adapted for positive identification of gene insertion and green fluorescent protein fusion, BioTechniques 25, 438-440, 442, 444.

Zhao, Y., Yu, M., Chen, M., Elder, R.T., Yamamoto, A., Cao, J., 1998b. Pleiotropic effects of HIV-1 protein R (Vpr) on morphogenesis and cell survival in fission yeast and antagonism by pentoxifylline. Virology 246, 266–276.